



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 15/62, 15/16, C07K 7/06 C07K 7/20, A61K 39/00		A1	(11) International Publication Number: WO 92/19746 (43) International Publication Date: 12 November 1992 (12.11.92)
(21) International Application Number: PCT/AU92/00194			(74) Agents: SLATTERY, John, M. et al.; Davies Collison Cave, 1 Little Collins Street, Melbourne, VIC 3000 (AU).
(22) International Filing Date: 29 April 1992 (29.04.92)			(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent), US.
(30) Priority data: 76177/91 29 April 1991 (29.04.91)		AU	(72) Inventors; and (75) Inventors/Applicants (for US only) : COX, John, Cooper [AU/AU]; 365 Bacchus Marsh Road, Bullengarook, VIC 3437 (AU). EDWARDS, Stirling, John [AU/AU]; 14 May Street, Coburg, VIC 3058 (AU).
			Published <i>With international search report.</i>

(54) Title: RECOMBINANT IMMUNOCASTRATION VACCINE AND POLYPEPTIDES USEFUL FOR SAME

(57) Abstract

The present invention relates generally to an immunocastration vaccine and to recombinant polypeptides useful for same. More particularly, the present invention is directed to a vaccine composition comprising a recombinant polypeptide comprising an amino acid sequence corresponding to luteinizing hormone releasing hormone or an analogue thereof, one or more T-cell epitopes and a purification site. The present invention further contemplates a method for the immunological castration of animals.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	RO	Romania
CA	Canada	IT	Italy	RU	Russian Federation
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark	MG	Madagascar		
ES	Spain				

RECOMBINANT IMMUNOCASTRATION VACCINE AND POLYPEPTIDES
USEFUL FOR SAME

5 The present invention relates generally to an immunocastration vaccine and to recombinant polypeptides useful for same. More particularly, the present invention is directed to a vaccine composition comprising luteinizing hormone releasing hormone or an analogue thereof. The present invention further contemplates a method for the immunological castration of animals.

10

An alternative to surgical castration is active immunisation against luteinizing hormone releasing hormone (hereinafter referred to as "LHRH") or an analogue thereof. LHRH is released from the hypothalamus into the pituitary portal vessels. It has been suggested that LHRH binds to the 15 gonadotrophs of the anterior pituitary to stimulate secretion and release of luteinizing hormone (LH). These hormones act on the sex organs, ovary and uterus in females or testis in males to produce both sex hormones (either oestrogens or androgens) and also to allow cellular differentiation within sex organs leading to production of active gametes. Active immunisation against 20 LHRH is proposed to disrupt the communication between the hypothalamus and pituitary. This active immunisation may lead to complete inhibition of sexual function in both males and females. It is thought that the site at which antibodies to LHRH might inhibit reproductive function is in the hypophyseal portal blood vessels.

25

Although active immunisation against LHRH has been achieved in some animals, individual animals vary significantly in their response to immunisation. This is probably due in part to the size of the LHRH molecule and how it is presented to an animal's immune system.

30

In current vaccines, the naturally-occurring decapeptide of LHRH⁽¹⁾ or various modifications or truncations thereof⁽²⁾ is chemically coupled to a

- 2 -

suitable carrier protein which acts as a source of T-cell epitopes. However, there are a number of problems associated with this approach which are summarised as follows:

5 1. Selection of carrier protein:

A number of proteins are used as peptide carriers. These include diphtheria toxoid (DT), tetanus toxoid (TT), keyhole limpet haemocyanin, ovalbumin, bovine serum albumin and the like. The most successful proteins have been DT and TT, presumably because they contain powerful T-cell epitopes with broad species reactivities. All of these proteins are, however, reasonably expensive to produce at the level of purity required for vaccine production. Additionally, the toxins of DT and TT require chemical detoxification prior to use which adds a further cost and uses surface reactive groups which would otherwise be available for peptide conjugation.

15

2. Source of LHRH:

The naturally occurring decapeptide LHRH or one of various analogues thereof (e.g. the nonapeptide 2-10) can be used for coupling to the carrier. Peptide can be manufactured in commercial quantities by chemical or enzymic synthesis. However, both processes are subject to error in synthesis and significant levels of impurity can survive extensive purification procedures. The final cost of purified peptide, even in commercial quantities, is expensive and becomes a major component of vaccine costs.

25 3. Chemical coupling of LHRH to carrier protein:

There are various well documented chemical procedures whereby LHRH can be coupled to a carrier protein. The choice of procedure will be determined by availability of reactive groups on the carrier protein, available reactive groups on LHRH or the chosen analogue, and acceptability of chemicals to vaccine registration authorities and the efficiency of the coupling procedure. Regardless of the choice of carrier protein, peptide and coupling reagent, coupling efficiency of peptide is rarely better than 40% to 50% and

formation of aggregates is difficult to avoid.

There is a need, therefore, to develop an LHRH based vaccine which is highly pure, chemically fully definable, reproducible, cost effective in

5 production and efficacious. In accordance with the present invention, a suitable vaccine is prepared by the construction of a recombinant molecule readily capable of purification by the incorporation or existence of a purification site.

10 Accordingly, one aspect of the present invention is directed to a recombinant polypeptide comprising an amino acid sequence corresponding to luteinizing hormone releasing hormone (LHRH) or an analogue thereof, one or more T-cell epitopes and a purification site, said polypeptide capable of stimulating an immune response in an animal against LHRH.

15

The term "polypeptide" is used in its most general sense and includes a peptide. The polypeptide may or may not be glycosylated and/or may or may not be associated with other proteinaceous or non-proteinaceous material.

20 A "recombinant" includes polypeptides manufactured by the expression of a nucleic acid sequence in a eukaryotic or prokaryotic organism. Preferably, the polypeptide is produced in large quantities by, for example, fermentation and as a single molecule, not requiring further modification. However, the present invention also extends to the recombinant production of separate parts
25 of the polypeptide which are then coupled by natural, chemical or recombinant means.

The "purification site" may be naturally occurring within the polypeptide molecule such as a glutathione-binding site on glutathione-S-transferase
30 (GST;(3)). Other molecules with naturally occurring purification sites include pertussis toxin and toxoid which can be purified by affinity association with asialated glycoproteins (e.g. asialofetuin) and cholera B toxin which binds to

GMI ganglioside. Alternatively, the purification may be added to the molecule as part of the genetic engineering process. An example of the latter is the addition of hexa-histidine residues (4) to a suitable antigen such as the non-toxic diphtheria toxin molecule derived from the CRM197 mutant of

5 Corynebacterium diphtheria, to which an LHRH amino acid sequence would also be attached. The purification site of the present invention can be further characterized as a "biochemical" rather than "immunological" purification site.

The T-cell epitope of the recombinant polypeptide must be capable of

10 recognition by the animal species so that it can stimulate a T-cell dependent immune response. The polypeptide may contain a single epitope or more than one. The choice of the source of T-cell epitopes may vary depending on the animal to be vaccinated.

15 The LHRH amino acid sequence or its analogues (including its derivatives) may be represented once in the polypeptide or as tandem or multiple repeats within the polypeptide or at its N-terminal or its C-terminal end. Derivatives and analogues of LHRH include any single or multiple amino acid substitutions, deletions and/or additions to the naturally occurring

20 or synthetic (eg. recombinant) amino acid sequence and further include immunological relatives of naturally occurring LHRH. Such alterations to the amino acid sequence of LHRH may occur at the amino terminal or carboxy terminal ends of the peptide or may occur within the peptide molecule itself. The analogues and derivatives of LHRH contemplated herein may not

25 necessarily function as a LH stimulating factor but may nevertheless have antibodies raised against it which will cross react with naturally occurring LHRH. Furthermore, the range of analogues and derivatives encompassed by the present invention also includes chemical alterations to the amino acid residues such as amidation. For example, the present invention extends to

30 LHRH without C-terminal amidation. When the polypeptide comprises more than one LHRH amino acid sequence, the sequences may all be the same or correspond to different derivatives and/or analogues.

Where the LHRH amino acid sequence is represented more than once, it may be as a tandem or multiple repeat, with or without spacers, at intervals throughout the carrier molecule or at intervals throughout the carrier molecule and including within the sequence an enzymic cleavage site so that the 5 expressed molecule can be converted enzymically into a number of peptides each of which will contain T-cell epitopes derived from the carrier molecule and a C-terminal LHRH. The enzymes cleavage site can be engineered to yield free carboxy or amidated LHRH.

10 In any event, reference herein to "LHRH" includes all such analogues or derivatives of LHRH, represented singly or multiply in the polypeptide.

In a preferred embodiment, the recombinant polypeptide further comprises means for self-assembly such that the polypeptide can self-assemble 15 into virion like-structures (e.g. Hepatitis⁵ B core antigen (HepB)) or into filaments (e.g. Potyvirus⁶).

The fusion polypeptide of the present invention therefore provides a carrier molecule (e.g. GST) which may be an integral part of a vaccine.

20 Expression of DNA or other nucleic acid sequences to produce the polypeptides of the present invention may be done by conventional techniques and reference can conveniently be made to Sambrook, Fritsch and Maniatis, Molecular Cloning: A Laboratory Manual, 1989 Cold Spring Harbor 25 Laboratory, Cold Spring Harbor, New York, USA. The present invention extends to all nucleic acid sequences encoding the polypeptides of the present invention, to expression vectors containing same and to eukaryotic and/or prokaryotic organisms transformed therewith.

30 Another aspect of the present invention contemplates a recombinant immunocastration vaccine suitable for use in an animal and capable of stimulating the production of antibodies to LHRH in said animal, said vaccine

comprising a polypeptide containing an LHRH amino acid sequence or an analogue thereof, one or more T-cell epitopes and a purification site.

The polypeptide is as hereinbefore described.

5

By "vaccine" as used herein is meant a composition of matter capable of stimulating a humoral and optionally also a cell-mediated immune response and/or any combinations thereof.

10 The vaccine of the present invention may be administered parenterally, for example, by subcutaneous, intramuscular and/or intravenous injection. Administration may also be orally, nasally, or by adsorption through the skin by microsponges or a mini-pump either implanted in the animal or attached outside the animal. The vaccine may also be administered by expression of a
15 host microorganism carrying a nucleic acid sequence which, when expressed, encodes the polypeptide of the present invention.

Vaccine formulations may also comprise one or more pharmaceutically acceptable carriers and/or diluents and may also contain an adjuvant.

20 Examples of adjuvants which may be used are aluminium hydroxide, Freund's complete or incomplete adjuvants, DEAE dextran, levamisole, PCG and polyI:C or polyA:U. Particularly preferred adjuvants are materials which do not cause local inflammation. One example of such material is a mineral oil composition that includes bacterial cell wall material such as peptidoglycans or
25 a synthetic derivative of such a cell wall material. Such a synthetic material is known as muramyl dipeptide. Suitable adjuvants are further described in Cox & Coulter (1992)(7).

Reference can conveniently be made to Remington's Pharmaceutical Sciences, 17th Edition, Mack Publishing, Easton, Pennsylvania, U.S.A. for various aspects of vaccine composition preparation.

By "animal" in relation to the suitability of a target for the vaccine includes any animal capable of mounting an immune response and which produces LHRH. Preferably, the animal is a mammal. Even more preferably, the animal is a live stock or domestic animal such as dogs, cats, sheep, cattle, 5 horses, goats or pigs. The mammal may also be a companion animal or a wild animal held in captivity or in a state of freedom. The present invention further extends to humans.

Yet another aspect of the present invention contemplates a method for 10 the immunocastration of an animal comprising administering to said animal an antibody-stimulating effective amount of a polypeptide, said polypeptide comprising an LHRH amino acid sequence or analogue thereof, one or more T-cell epitopes and a purification site, for a time and under conditions sufficient for said animal to generate LHRH specific antibodies. These 15 antibodies will eventually result in the animal becoming immunocastrated. The latter condition is considered to occur when the level of LH is reduced to an extent where the level of testosterone or progesterone is substantially lowered. In accordance with this method, the LHRH amino acid sequence, T-cell epitope and purification site have the same meaning as hereinbefore 20 described. The route of administration may be as described above. Furthermore, the recombinant immunocastration vaccine may be expressed in a microorganism which is, or can become part of, the normal flora of the animal. The engineered microorganism would then secrete an effective 25 amount of the recombinant vaccine. The microorganism may also be mutated such that expression of the vaccine can be induced or repressed depending on environmental stimuli such as diet.

The vaccine may be administered once or multiple administrations may 30 be given. For example, following initial vaccination, booster injections or administrations may be given every 4 to 10 weeks or whenever appropriate for the animal concerned. The age of the animal to be vaccinated will vary depending on the animal but may range from 8 to 50 weeks for calves to 8 to

24 weeks for lambs.

The carrier specifically exemplified herein uses GST derived from Schistosoma japonicum. However, other GST molecules can be used as well 5 as other carrier molecules having purification properties (e.g. pertussis toxoid and cholera B toxin). The carriers of the present invention must also assist in the stimulation of an immune response.

The immunocastration vaccine of the present invention, therefore, is 10 cost effective, chemically defined, efficacious, reproducible and highly pure and, therefore, provides a valuable and useful means for castrating animals.

In summary, the present invention provides an immunocastration vaccine with the following combined preferred characteristics:

15

- (i) Low cost of production
- (ii) High purity
- (iii) Fully characterisable molecular entity
- (iv) High efficacy.

20 To achieve the above, and in particular (i) and (iii), it is preferable to construct a recombinant molecule which can be produced by convenient means such as by microbial fermentation. In a most preferred embodiment of the present invention, glutathione-S-transferase (GST) molecule is selected and an 25 LHRH decapeptide expressed at the C-terminal end of this protein was engineered. GST on its own or as a fusion protein can be readily purified on a glutathione affinity column. Such purification is inexpensive, highly effective and non-destructive. In the present application, it has been realised that rather than being undesirable or at best irrelevant, the GST molecule can 30 become an integral component of an immunocastration vaccine by supplying T epitopes which are a natural component of its polypeptide structure. Thus, a recombinant GST-LHRH molecule meets (i) to (iii) of the above criteria in

that it can:

- (i) be produced as a single molecule in an E. coli expression system
- (ii) be purified to high purity by use of its glutathione binding site
- 5 (iii) be fully characterisable, thus eliminating batch to batch variations.

The present invention is further described by reference to the following non-limiting Figures and Examples.

10 In the Figures:

Figure 1A is a photographic representation showing screening of transformed colonies for IPTG-induced expression of the GST/LHRH hybrid protein by Western blot. (8)

15

Two colonies (Nos. 1 and 16) arising from the transformation of E.coli with ligated pGEX1/LHRH were screened with sheep anti-LHRH antiserum.

20 Lanes 1 to 9 contained samples of supernatants from lysed cells of:

Colony No. 16 at 0,3,4,0 and 3 hours after addition of IPTG (lanes 1 to 5, respectively).

25 Colony No. 16 at 0 and 3 hours where no IPTG was added (lanes 6 and 7, respectively).

Colony No. 1 at 0 and 3 hours after addition of IPTG (lanes 8 and 9, respectively).

30 Thus Colony No. 16 was positive for GST/LHRH expression whereas Colony No. 1 was not. Colony No. 16 was chosen for further analysis.

- 10 -

Figure 1B is a photographic representation of a Coomassie stained gel corresponding to the Western blot.

Figure 2 is a photographic representation of a Coomassie-stained gel 5 showing the purification of the recombinant vaccine by glutathione column chromatography.

Samples 1 to 7:

1. Cell sonicate.
2. Clarified supernatant.
- 10 3. Unadsorbed material from glutathione.
4. Blank
- 5,6 & 7. 10, 5 & 2 μ l sample of eluted peak.

EXAMPLE 1

15 A) Vaccine Construction

The DNA sequence corresponding to LHRH was inserted into the multiple cloning site of the pGEX1 plasmid vector (5) by the following method:

20 Two DNA oligomers each of 38 residues were synthesised and annealed to form a double stranded oligomer which, upon insertion into the multiple cloning site of the p-GEX1 coded for the LHRH amino acid sequence QHWSYGLRPG in frame with the GST sequence. This double stranded 25 DNA oligomer had the following specific characteristics:

(i) It contained single stranded overhanging ends corresponding to a Bam H1 cohesive sequence at the 5' end and a Eco R1 cohesive 30 sequence at the 3' end. This ensured that the DNA oligomer was inserted into Bam H1/Eco R1 cleaved pGEX1 in the desired orientation.

(ii) The termination codon TGA immediately followed the sequence coding for the LHRH terminal G amino acid, ensuring this amino acid was the C-terminal residue of the GST/LHRH hybrid protein.

5 The annealed oligomer was ligated into Bam H1/Eco R1 cleaved pGEX1 by conventional means. The ligation mix was transformed into competent E. coli K12 strain DH5 α cells. Single colonies were selected by overnight growth on Luria agar containing 100 μ g/ml carbenicillin. All further growth of transformed cells was carried out in Luria broth containing 100
10 μ g/ml carbenicillin (LBC). To test for expression of a GST/LHRH hybrid protein, overnight cultures of colony picks grown in LBC at 37 °C were diluted 1:10 in fresh LBC and grown for 1 to 2 hours at 37 °C. To induce GST production, IPTG was added to 0.1 mM and growth allowed to proceed for a further 3 to 4 hours at 37 °C. Cells were pelleted, lysed by sonication and
15 clarified. Supernatant samples were subjected to SDS-PAGE and Coomassie-stained gels and Western blots prepared. Blots were screened using a sheep anti-LHRH antiserum. The Western blot in Fig. 1A shows the appearance of a band recognised by the antiserum 3 and 4 hours after induction with IPTG. This band corresponds to a major Coomassie-stained band on the
20 corresponding gel (Fig. 1B) which is at the expected molecular weight for a GST protein containing a single copy of LHRH.

B) Vaccine Production

25 For large scale preparation of the GST/LHRH protein, shaker flasks were grown and incubated under the conditions outlined above and a cell sonicate prepared as described. The supernatant was purified by glutathione-agarose column chromatography. The GST/LHRH hybrid protein was eluted from the column using 5 mM reduced glutathione in 10 mM Tris HCl pH 8.2.
30 Fig. 2 shows the result of a glutathione column purification. In one step GST/LHRH was eluted from the column in a highly pure state in a yield estimated at 30 μ g/ml of the original broth culture.

C) Vaccine Formulation, Immunisation and Testing

Vaccines were adjuvanted with FCA by addition of a third of a volume of conjugate or GST/LHRH in PBS at one time to the required volume of

5 FCA. The mixture was then emulsified using a Silverson Heavy Duty Laboratory mixer at maximum speed for one minute. This process was performed another two times. The emulsion formed was tested by placing a drop of emulsion on the surface of water. The emulsion was considered stable if the drop remained intact for at least two minutes.

10

Swiss male mice were used when they reached a mass of > 20gm (approximately 8 weeks of age). They received two doses three weeks apart and were bled two weeks after the second dose. Testes were removed seven weeks after the second dose.

15

D) Measurement of Anti-LHRH Titres

Dilutions of sera (200ul) in 0.1M NaCl, 10mM sodium phosphate, 0.1% gelatin, 0.1% sodium azide pH 7.38 were added to 3 H-LHRH (NEN) (100ul, ca 20 10000dpm) and 100ul of 0.5% human gamma globulin for 48 hours at 4 °C. Cold 18% PEG 6000 (1ml) was added and the tubes were vigorously mixed for 3 minutes and centrifuged at 3000g for 30 minutes. The supernatants were aspirated and the precipitate redissolved and the radioactivity measured. Anti-LHRH titres in sera samples were calculated by plotting the logit of the 25 percentage specific binding versus the log of the serum dilution and interpolating to the dilution giving 30% binding. Titres were expressed as the reciprocal of this serum dilution.

Mouse testes were weighed after mice were killed. Normal mice had 30 testes weights in the range from 0.17-0.32 gm. Male mice were assessed for fertility and sexual activity by caging a test male with 2 mature female mice. After 14-18 days females were killed and the number of foetuses counted.

Normal male control mice always produce 6-12 foetuses per female. Treated mice were considered fertile if they produced any foetuses in either female. All testes were removed and weighed. Mice having testes weights less than 0.12 gm were invariably infertile while those having testes weights of 0.13-0.16 gm showed reduced fertility (1-6 foetuses in at least 1 female). It is concluded that testes weight of mice when mice had been treated as above, could be used to assess fertility. Any mouse with a testes weight of <0.12gm was deemed to be infertile.

10 E) Immunocastration of Mice

Three groups of mice were vaccinated in the following protocol:

Group 1: 2 doses each 1mg/ml GST-LHRH in FCA
Group 2: 2 doses each 0.1 mg/ml GST-LHRH in FCA
15 Group 3: 2 doses each DT/LHRH in FCA

The results are shown in Table 1.

F) Immunocastration of Cats

20 Four groups each of six cats were dosed with LHRH - protein conjugates as detailed in Table 2. The first three were different batches of the same chemical conjugates of LHRH and diphtheria toxoid, the fourth a recombinant GST/LHRH as described above. The adjuvant used for all conjugates was a water in oil emulsion containing DEAE dextran. Cats were 25 dosed at week zero and four then bled at week eight. These results show immunisation of cats with GST-LHRH give rise to anti-LHRH antibodies which indicates that the recombinant vaccine is at least as good as a chemically synthesised vaccine.

- 14 -

TABLE 1
ANTIBODY TO LHRH AND TESTES SIZE FOR MICE IMMUNISED
WITH GST-LHRH VACCINE

GROUP 1		GROUP 2		GROUP 3	
α (LHRH titre	Testis weight	α (LHRH titre	Testis weight	α (LHRH titre	Testis weight
450	0.03*	170	0.21	1180	0.07*
350	0.12*	125	0.16	865	0.06*
310	0.20	<20	0.18	805	0.23
175	0.23	<20	0.19	800	0.04*
170	0.16	<20	0.20	740	0.15
155	0.19	<20	0.22	625	0.18
75	0.24	<20	0.22	460	0.08*
<20	0.17	<20	0.23	225	0.18
<20	0.18	<20	0.24	<20	0.19
<20	0.26	-	-	<20	0.22

* Immunocastrated

TABLE 2
ANTIBODY TITRES IN CATS DOSED WITH CHEMICAL AND
RECOMBINANT LHRH CONJUGATES

GROUP 1 DT-LHRH	GROUP 2 DT-LHRH	GROUP 3 DT-LHRH	GROUP 4 GST-LHRH
4,950	471	725	1,955
1,831	381	530	225
286	255	243	193
265	118	130	162
232	112	76	136
215	<20	<20	50

Example 2**Construction of Recombinant GST Containing Tandem Repeats of LHRH**

Multimers of the DNA sequence corresponding to LHRH were inserted
5 into the multiple cloning site of pGEX1 by the following method:

(i) Two DNA oligomers (58 and 61) each of 30 bases corresponding to the coding sequence for the LHRH amino acid sequence QHWSYGLRPG were synthesized and annealed to form a double-stranded oligomer
10 (58/61). This was then treated with T4 polynucleotide kinase. Oligomer 58/61 contained four base single-stranded cohesive ends enabling polymerisation of the oligomer in a head to tail fashion. Polymerisation of 58/61 was carried out using ligase to produce a mixture of multimers containing tandem repeats. This mixture was
15 subjected to electrophoresis on 8% polyacrylamide gels and bands corresponding to multimers containing particular numbers of tandem repeats identified by comparison with standard molecular weight markers. The bands were eluted from the gel and ligated to a T4 polynucleotide kinase-treated eight base single-stranded adaptor
20 (oligomer 57) to form oligomers designated 57multi58/61. The adaptor comprised four bases corresponding to a BamH1 cohesive end and four bases complementary to the 5' four base single-stranded overhand of polymerised 58/61.

25 (ii) In a separate experiment two single-stranded oligomers of 10 and 18 bases (oligomers 59 and 60, respectively) were synthesized and annealed. Oligomer 59/60 contained a 5' four base single-stranded overhang complementary to the 3' four base single-stranded overhand of oligomers 57multi58/61 above and a 3' EcoR1 cohesive end. It also
30 contained a TGA stop codon positioned such that when 59/60 was ligated to any variant of 57multi58/61 the stop codon immediately

followed the sequence coding for the terminal G amino acid of LHRH of the terminal repeat of 58/61.

5 (iii) T4 polynucleotide kinase-treated oligomer 59/60 was ligated to BamH1/EcoR1 cleaved pGEX1. The ligated product was purified by stranded methods and ligated to the 57multi58/61 oligomers. The ligation mixtures were transformed into E.coli strain DH5 α . Antibiotic selection of transformants and screening for expression of GST containing multimers of LHRH were carried out as described in Example 1.

10

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications which fall within its spirit and scope. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

REFERENCES:

1. Schanbacher, 1986 in "Immunological aspects of reproduction in mammals" (DB Crighton, ed. Butterworths, London, p345.)
2. U.S. Patent No. 4 608 251
Australian Patent Application No. 22755/88
Australian Patent Application No. 79453/87
3. International Patent Application No. WO 88/09372
4. Australian Patent Application No. 12709/88
5. Smith and Johnson, Gene 67: 31-40, 1988
6. Jagadish et al.(1992) J. Gen. Virol. 72, 1543-1550.
7. Cox and Coulter, in Animal Parasite Control
8. Burnette (1981) Anal. Biochem 112, 195.

CLAIMS:

1. A recombinant polypeptide comprising an amino acid sequence corresponding to luteinizing hormone releasing hormone (LHRH) or an analogue thereof, one or more T-cell epitopes and a purification site, said polypeptide capable of stimulating an immune response in an animal against LHRH.
2. The recombinant polypeptide according to claim 1 wherein said amino acid sequence corresponds to two or more copies of LHRH and/or an analogue thereof.
3. The recombinant polypeptide according to claim 1 wherein the purification site is naturally occurring within the polypeptide molecule.
4. The recombinant polypeptide according to claim 3 wherein the purification site is a glutathione-binding site on glutathione-S-transferase.
5. The recombinant polypeptide according to claim 3 wherein the purification site is located on pertussis toxin, pertussis toxoid or cholera B toxin.
6. The recombinant polypeptide according to claim 3 or 4 wherein the purification site comprises hexa-histidine residues.
7. The recombinant polypeptide according to claim 1 wherein said amino acid sequence further comprises residues permitting the polypeptide to self-assemble into a virion-like structure.
8. The recombinant polypeptide according to claim 1 wherein the animal is a mammal.

- 19 -

9. The recombinant polypeptide according to claim 8 wherein the mammal is a human, livestock animal or domestic animal.
10. The recombinant polypeptide according to claim 8 wherein the mammal is a companion animal or wild animal held in captivity or in a state of freedom.
11. The recombinant polypeptide according to claim 1 wherein the T-cell epitope occurs as a normal component in said polypeptide.
12. A recombinant immunocastration vaccine suitable for use in an animal and capable of stimulating the production of antibodies to luteinizing hormone releasing hormone (LHRH) in said animal, said vaccine comprising a polypeptide containing an LHRH amino acid sequence or an analogue thereof, one or more T-cell epitopes and a purification site and one or more pharmaceutically acceptable carriers and/or diluents.
13. The recombinant immunocastration vaccine according to claim 12 wherein said polypeptide comprises two or more copies of an LHRH amino acid sequence and/or an analogue thereof.
14. The recombinant immunocastration vaccine according to claim 12 wherein the purification site is naturally occurring within the polypeptide molecule.
15. The recombinant immunocastration vaccine according to claim 14 wherein the purification site is a glutathione-binding site on glutathione-S-transferase.
16. The recombinant immunocastration vaccine according to claim 14 wherein the purification site is located on pertussis toxin, pertussis toxoid or cholera B toxin.

- 20 -

17. The recombinant immunocastration vaccine according to claim 12 wherein the purification site is hexa-histidine.
18. The recombinant immunocastration vaccine according to claim 12 wherein the polypeptide further comprises residues permitting the polypeptide to self-assemble into a virion-like structure.
19. The recombinant immunocastration vaccine according to claim 12 wherein the animal is a mammal.
20. The recombinant immunocastration vaccine according to claim 19 wherein the mammal is a human, livestock animal or domestic animal.
21. The recombinant immunocastration vaccine according to claim 19 wherein the mammal is a companion animal or wild animal held in captivity or in a state of freedom.
22. The recombinant immunocastration vaccine according to claim 12 wherein the T-cell epitope occurs as a normal component of the polypeptide.
23. The recombinant immunocastration vaccine according to claim 12 further comprising an adjuvant.
24. The recombinant immunocastration vaccine according to claim 23 wherein the adjuvant does not cause inflammation.
25. A method for the immunocastration of an animal comprising administering to said animal an immune response stimulating effective amount of a recombinant polypeptide, said recombinant polypeptide comprising an amino acid sequence corresponding to luteinizing hormone releasing hormone (LHRH) or an analogue thereof, one or

more T-cell epitopes and a purification site, for a time and under conditions sufficient for said animal to generate LHRH-specific antibodies.

26. The method according to claim 25 wherein said amino acid sequence corresponds to two or more copies of LHRH and/or an analogue thereof.
27. The method according to claim 25 wherein the purification site is naturally occurring with the polypeptide.
28. The method according to claim 27 wherein the purification site is a glutathione binding site on glutathione-S-transferase.
29. The method according to claim 27 wherein the purification site is located on pertussis toxin, pertussis toxoid or cholera B toxin.
30. The method according to claim 25 wherein the purification site comprises hexa-histidine residues.
31. The method according to claim 25 wherein said amino acid sequence further comprises residues permitting the polypeptide to self-assemble into a virion-like structure.
32. The method according to claim 25 wherein the animal is a mammal.
33. The method according to claim 32 wherein the mammal is a human, live-stock animal or domestic animal.
34. The method according to claim 32 wherein the mammal is a companion animal or a wild animal held in captivity or in a state of freedom.

- 22 -

35. The method according to claim 25 wherein administration is by subcutaneous, intramuscular, intravenous, oral or nasal administration.
36. The method according to claim 25 wherein the administration is by expression of a nucleic acid molecule encoding the polypeptide in a microorganism located in an animal.
37. The method according to claim 25 wherein the T-cell epitope occurs as a normal component in the polypeptide.

1/3

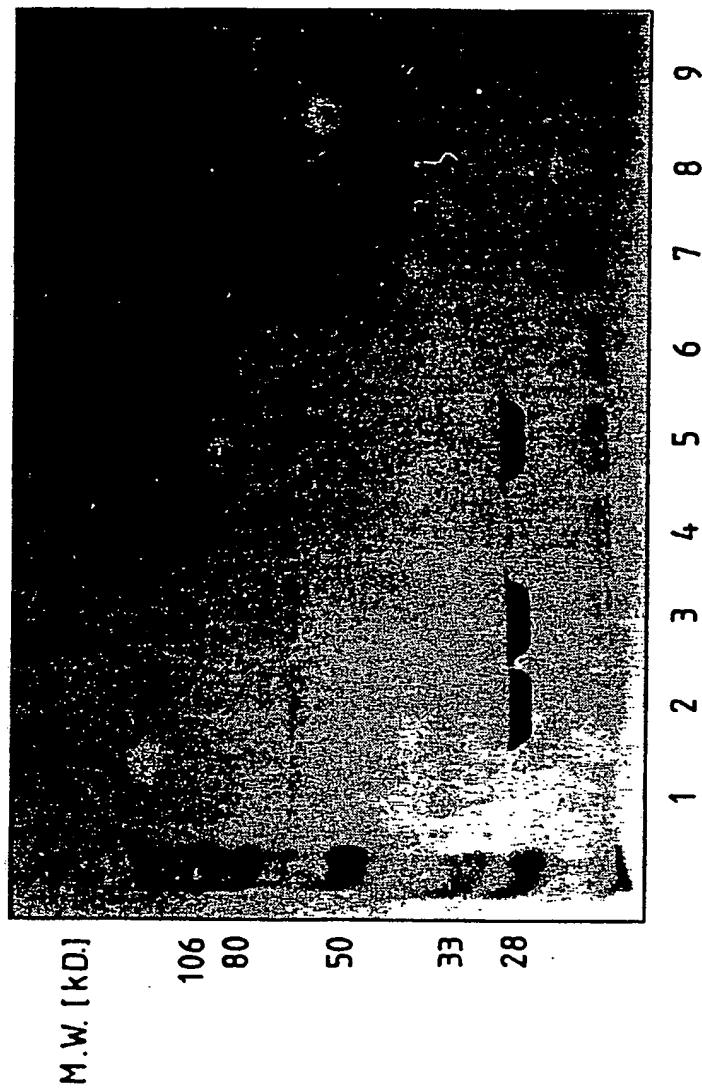


Fig. 1A.

2/3

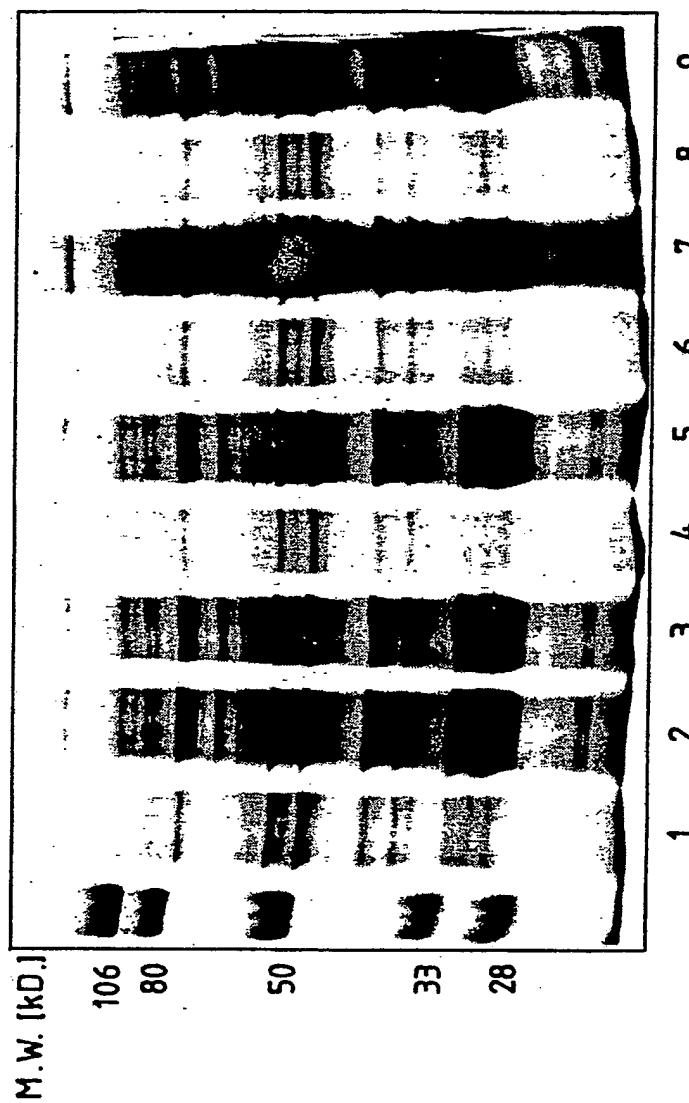


Fig. 1B.

3/3

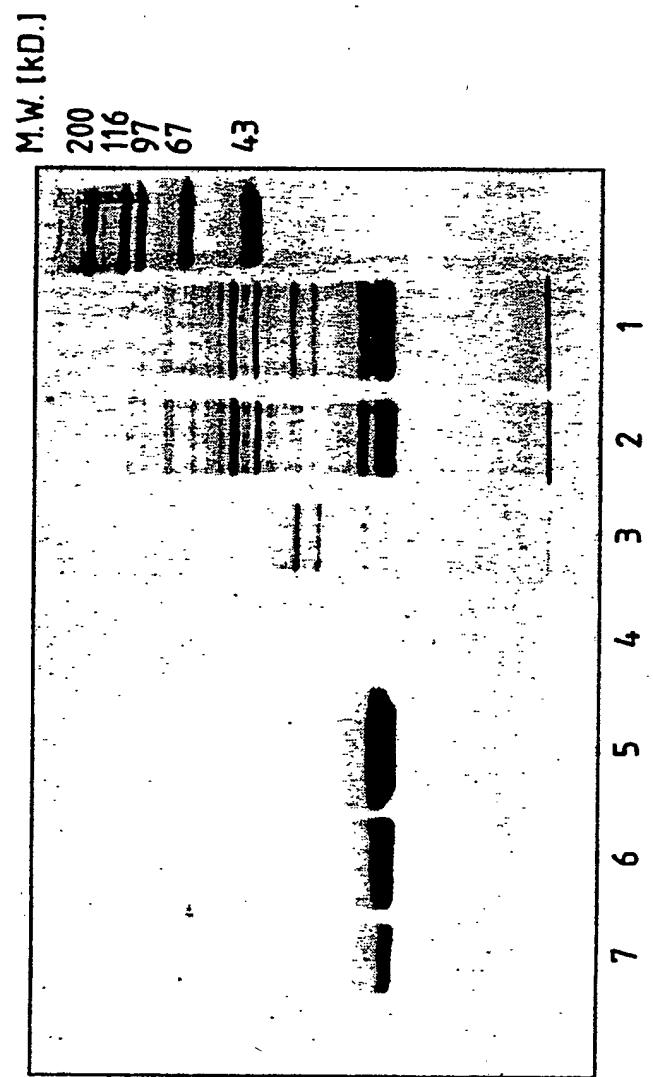


Fig. 2.

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶								
According to International Patent classification (IPC) or to both National Classification and IPC Int. Cl. ⁵ C12N 15/62, C12N 15/16, C07K 7/06, C07K 7/20, A61K 39/00								
II. FIELDS SEARCHED								
<table border="1"> <thead> <tr> <th colspan="2">Minimum Documentation Searched⁷</th> </tr> <tr> <th>Classification System</th> <th>Classification Symbols</th> </tr> </thead> <tbody> <tr> <td>IPC Chem. Abstr.</td> <td> WPAT keywords 1. Immunocastration 2. LHRH or luteinizing(hormone) releasing(hormone and peptide or polypeptide and antibody) </td> </tr> </tbody> </table>			Minimum Documentation Searched ⁷		Classification System	Classification Symbols	IPC Chem. Abstr.	WPAT keywords 1. Immunocastration 2. LHRH or luteinizing(hormone) releasing(hormone and peptide or polypeptide and antibody)
Minimum Documentation Searched ⁷								
Classification System	Classification Symbols							
IPC Chem. Abstr.	WPAT keywords 1. Immunocastration 2. LHRH or luteinizing(hormone) releasing(hormone and peptide or polypeptide and antibody)							
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸								
AU: IPC 5: C12N 15/62, C12N 15/16 BIOT: LHRH or luteinizing(hormone) releasing(hormone)								
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹								
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate of the relevant passages ¹²	Relevant to Claim No ¹³						
X	AU,B,61782/90 (622044) (BIOTECHNOLOGY AUSTRALIA PTY LTD) 7 March 1991 (07.03.91). See whole document;	1-3, 8-14, 19-27, 32-35, 37						
X	AU,B,79453/87 (610526) (PROTEUS BIOTECHNOLOGY LTD) 8 December 1988 (08.12.88). See page 6 lines 15-18, claims;	1-3, 8-14, 19-27, 32-35, 37						
X	AU,B,58612/86 (594059) (BIOTECHNOLOGY AUSTRALIA PTY LTD) 20 November 1986 (20.11.86). See pages 32-35, claims 36-43, 47, 57.	1,3,8,9,10,11,12,14, 19,20,21,22,23,24, 25,27,32-35,37						
(continued)								
* Special categories of cited documents : ¹⁰ "A" Document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed								
" "T" Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art " & " document member of the same patent family								
IV. CERTIFICATION								
Date of the Actual Completion of the International Search 12 August 1992	Date of Mailing of this International Search Report 17 Aug 1992 (17.08.92)							
International Searching Authority AUSTRALIAN PATENT OFFICE	Signature of Authorized Officer K AYERS <i>Karen Ayers</i>							

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	AU,B,28438/89 (619239) (BUNGE (AUSTRALIA) PTY LTD) 13 July 1989 (13.07.89), see pages 4, 5, 10 and claims;	1,2,3, 8-14, 19-27 32-35, 37
X	AU,B,22755/88 (605358) (UNIVERSTITY OF SASKATSCHEWAN) 20 April 1989 (20.04.89). See whole document;	1-3, 8-14, 19-27 32-35, 37

V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claim numbers ..., because they relate to subject matter not required to be searched by this Authority, namely:
2. Claim numbers ..., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. Claim numbers ..., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4a

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this international application as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- The additional search fees were accompanied by applicant's protest.
- No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 92/00194

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Member				
AU 90/61782	CA 2040441 WO 9102799	EP 446313	JP 4502163		
AU 87/79453	EP 293530 GB 2196969	GB 8713240 GR 89300071		GB 8723072	
AU 86/58612	CN 86103835 ES 554969 JP 62503031 WO 8606635	DK 164/87 FI 870140 NO 870148 ZA 8603612	EP 222835 IL 78775 NZ 216162		
AU 89/28438	CN 1034581 JP 2153000	DK 81/89 NO 890115	EP 324625 ZA 8900257		
AU 88/22755	AT 74516 EP 309863 US 4975420	DE 3869892 JP 2000113 ZA 8807304	DK 5434/88 NZ 226348		